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Detection of β-Lactamase Reporter Gene Expression by Flow Cytometry

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Background: Flow cytometry of gene expression in living cells requires accurate, sensitive, nontoxic fluorescent indicators capable of detecting transcription of specific genes. This is typically achieved by using genes that encode fluorescent proteins or enzymes coupled to promoters of interest. The most commonly used reporters are green fluorescent protein and β-galactosidase (*lacZ*). In this study, we characterized the performance of a cell-permeant, ratiometric, β-lactamase substrate, coumarin cephalosporin fluorescein (CCF2/AM). We compared its characteristics with that of the β-galactosidase/fluorescein di-β-D-galactopyranoside reporter system.
Methods: Jurkat cell lines were generated for β-lactamase and β-galactosidase reporters with the use of similar plasmid constructs. Rare event flow cytometric detection for the β-galactosidase and β-lactamase reporters were assayed by using mixed populations of negative (WT) and positive (constitutively expressing) cells for each reporter. To determine sensitivity at low reporter copy number, we measured the activity of an unstimulated inducible promoter and detected positive events as a function of substrate incubation time. Technical issues related to data processing and optical configuration are also presented.
Results: The low population coefficients of variation afforded by ratiometric detection of the β-lactamase system improved the statistical performance of the assay in comparison with a single-dye, intensity-based assay, leading to markedly improved detection for low copy number and rare events. At low levels of gene expression, β-lactamase was detected with approximately 10-fold higher confidence than was β-galactosidase. In rare event detection experiments, cells expressing high levels of β-lactamase proteins were reliably detected at frequencies of $1:10^6$ compared with about $1:10^4$ for β-galactosidase.
Conclusion: The ratiometric fluorescence readout of the β-lactamase system based on fluorescence resonance energy transfer allowed more sensitive and accurate detection of gene expression than the currently available β-galactosidase substrates. Further, the cell-permeant nature of the substrate improved experimental convenience. These properties facilitated cell engineering and enabled a variety of applications including selection of rare cells from large populations and measurement of low-expressing or downregulated genes. Cytometry Part A 51A:68-78, 2003. © 2003 Wiley-Liss, Inc.

Key terms: flow cytometry; fluorescence resonance energy transfer; β-lactamase; β-galactosidase; lacZ; CCF2; rare event; reporter gene; diode laser

The ability to measure gene expression in single living cells is important for studying cell function, with applications in cell engineering, signal transduction, and functional genomics. The ability to use flow cytometry to isolate single-cell clones based on gene expression is a key utility for many of these applications. The two most commonly used reporter genes compatible with flow cytometry are green fluorescent protein (GFP) (1) and *lacZ*, the gene encoding β-galactosidase (2-5), but both have drawbacks. For example, GFP is a relatively insensitive reporter due to lack of enzymatic amplification, and it is estimated that 50,000-100,000 copies per cell of protein are the lower limit for reliable detection (6). β-Galactosidase (β-gal) is more sensitive than GFP because it is an enzyme, but it has the disadvantages of being endogenously expressed in many mammalian cells and the currently available substrate, fluorescein di-β-D-galactopyranoside (FDG), requires hypotonic shock for efficient loading into cells (2,7). Further, the fluorescent hydrolysis product is poorly retained inside living cells, requiring that cells be kept on ice , thus limiting the time window for substrate conver-

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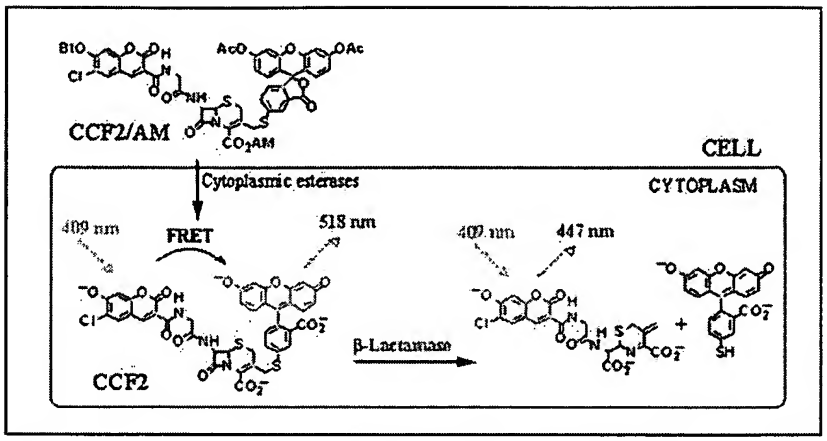

FIG. 1. Schematic diagram of coumarin cephalosporin fluorescein (CCF2) readout in living cells. CCF2/AM, the esterified form of CCF2, is passively loaded into living cells at room temperature in isotonic buffer at loading concentrations typically in the range of 1-2 μM. After entering the cytoplasm of the cell, the substrate is de-esterified by cellular esterases yielding CCF2 with a net −4 charge. Fluorescence resonance energy transfer (FRET) is disrupted upon cleavage of CCF2 by β-lactamase, causing a change from green to blue fluorescence upon violet or ultraviolet excitation.

sion. There is also evidence of active extrusion of fluorescein-galactoside by certain cell types (8).

Coumarin cephalosporin fluorescein (CCF2/AM; PanVera LLC, Madison, WI) is a membrane-permeant, ratiometric β-lactamase substrate applicable for flow cytometry (9,10). CCF2 is passively loaded into living mammalian cells as an esterified form (CCF2/AM). Intracellular processing of the esterified form yields a well-retained fluorescent substrate. CCF2 is composed of a fluorescein and coumarin linked by a cephalosporin bridge, which is cleaved in the presence of the β-lactamase enzyme. With violet or ultraviolet (UV) excitation, the intact CCF2 fluoresces green due to fluorescence resonance energy transfer (FRET) between the coumarin and the green-emitting fluorescein. In the presence of β-lactamase, substrate cleavage allows the coumarin to emit blue fluorescence. A schematic representation of the CCF2 substrate mechanism and readout is outlined in Figure 1. By measuring light output in the blue and green channels, one can obtain a ratiometric readout that is minimally affected by variations in cell size, cell number, cell-to-cell loading heterogeneity, probe concentration, and optical path.

In this study we evaluated and compared the performance of β-lactamase/CCF2 and β-gal/FDG reporter systems by using matched cell lines expressing similar levels of reporter gene. We also report on the use of a low-cost diode laser as an alternative light source to excite the coumarin-containing substrate.

MATERIALS AND METHODS

CCF2/AM Substrate Ester Loading for β-Lactamase Detection

Jurkat cells were collected during log phase growth and centrifuged (3 min at 207*g*). The supernatant was discarded and cells were resuspended in an isotonic loading buffer containing 1 μM of CCF2/AM (from 1 mM stock in dimethyl sulfoxide) in Hank's Balanced Salt Solution (HBS) supplemented with 25 mM of HEPES, pH 7.4. Cells were gently mixed for 1 h at room temperature. Samples were then centrifuged (3 min at 207*g*), resuspended in HBS (with 1 g/l of glucose and 25 mM of HEPES, pH 7.4), and placed on ice until use.

Flow Cytometry

For all experiments for detection of rare events and of low copy number of reporter enzyme, flow cytometry was conducted with Becton Dickinson (BD; San Jose, CA) FACSVantage flow cytometers. CCF2 can be analyzed with any flow cytometer equipped with a violet or UV (351-364 nm) excitation source and appropriate light collection optics with suitable wavelengths. Although the CCF2 readout works well with UV excitation, violet excitation yields optimal performance. For β-lactamase experiments, 60 mW of 413-nm excitation (Innova 302C krypton laser, Coherent, San Jose CA) was used with a 500-nm dichroic filter separating a 460/50-nm (CCF2 blue fluorescence) and a 535/40-nm bandpass filter (CCF2 green fluorescence; optical filters from Chroma Technology, Brattleboro, VT). For all β-gal experiments, 100 mW of 488-nm excitation (Enterprise laser, Coherent) was used with a 530/30-nm bandpass emission filter for FDG fluorescence detection.

The β-lactamase/CCF2 readout was also analyzed on a modified BD LSR. The LSR (standard configuration) is equipped with a 20-mW air-cooled argon (488 nm) laser as the primary beam and an 8-mW HeCd UV (325 nm) laser as the secondary excitation source. For our analysis of CCF2 on the BD LSR, we turned off the HeCd laser and installed a 5-mW violet (404 nm) diode laser (Power Technology, Inc., Little Rock, AR). CCF2 fluorescence on the BD LSR was collected with the use of 460/50-nm (CCF2 blue fluorescence) and 520/10-nm (CCF2 green fluorescence) bandpass filters separated by a 490-nm longpass dichroic filter (the optical configuration for fluorescence emission collection on the BD LSR is optimized for shorter wavelength dyes such as Indo-1 and likely would perform better for CCF2 with further optical optimization for this fluorophore).

Table 1
Expression Levels of Reporter Enzyme in Stable Clonal Cell Lines[*]

Cell line	Plasmid or reporter	Antibiotic selection in plasmid	Stimulation conditions	No. copies of functional reporter enzyme/cell detected[a]
WT Jurkat	None (used as background for reporter enzyme copy number determination)	None	None	10-40 (β-lactamose monomers: background)
C84 Jurkat	p3 × NFAT-IL2β-lactamase	Zeocin	Unstimulated	70-160 (β-lactamose monomers)
C84 Jurkat	p3 × NFAT-IL2β-lactamase	Zeocin	100 nM PMA + 1 μM thapsigargin. 5 h at 37°C, 5% CO_2, 90% humidity	33,000-40,000 (β-lactamose monomers)
CMV-β-lactamase Jurkat	pcDNA3-CMV-β-lactamase	Neomycin	Constitutive	15,000-20,000 (β-lactamose monomers)
WT Jurkat	None (used as background for reporter enzyme copy number determination)	None	None	8-10 (β-gal tetramers: background)
C37 Jurkat	p3 × NFAT-IL2-lacZ	Zeocin	Unstimulated	11-15 (β-gal tetramers)
C37 Jurkat	p3 × NFAT-IL2-lacZ	Zeocin	100 nM PMA + 1 μM thapsigargin, 5 h at 37°C, 5% CO_2, 90% humidity	6,400-8,000 (β-gal tetramers)
CMV-lacZ Jurkat	pcDNA3-CMV-lacZ	Hygromycin	Constitutive	7,400-8,400 (β-gal tetramers)

[*]CMV, cytomegalovirus; IL2, interleukin 2; NFAT, nuclear factor of activated T cells; PMA, phorbol myristate acetate.
[a]Before subtraction of background readout of negative cells.

Generation of Jurkat Cell Lines

Wild-type Jurkat cells were purchased from American Tissue Culture Collection (Rockville, MD) and maintained in growth medium (RPMI 1640, Gibco/Invitrogen, Carlsbad, CA) supplemented with the following: 10% heat-inactivated fetal bovine serum, 0.1 mM of nonessential amino acids (Gibco/Invitrogen), 1 mM of sodium pyruvate, 1× Pen/Strep, 55 μM of 2-mercaptoethanol, 2 mM of L-glutamine, and 25 mM of HEPES buffer. Two pairs of cell lines were generated. For measuring low-level gene expression, we generated cell lines containing *lacZ* or β-lactamase under the control of an inducible nuclear factor of activated T cells (NFAT) construct and measured the activity of cells in the uninduced state. For rare event experiments, we generated a pair of stable clones in which *lacZ* or β-lactamase are driven by a constitutively active cytomegalovirus (CMV) promoter. The plasmid constructs used and characteristics of these cell lines are summarized in Table 1.

For all transfections, WT Jurkat cells were fed with fresh growth medium 1 day before transfection. After washing once in Cytomix (11) buffer, 2×10^6 cells were pelleted and resuspended in 0.5 mL of Cytomix buffer supplemented with 15 μg of plasmid DNA. This mixture was electroshocked in a 2-mm cuvette placed in a Gene Pulser (Bio-Rad, Hercules, CA) at 290 V/960 μF. After electroporation the cells were transferred and cultured for 48 h in RPMI 1640 containing 10% fetal bovine serum. After antibiotic selection for about 3 weeks, stable single-cell clones were sorted with flow cytometry, as described above.

Quantitation of Reporter Enzyme Copy Number in Stable Cell Lines

To determine the average copy number of functional reporter enzyme per cell, rates of substrate hydrolysis were calculated from biochemical assays of cell lysates by using purified β-lactamase (gift from S. Mobashery) or purified β-gal (Calbiochem, San Diego, CA) as a reference standard. β-Lactamase cell enzyme determinations were done as previously described (12). For β-gal determinations, a similar methodology was employed with 10 μM of 4-methylumbelliferyl-β-D-galactopyranoside substrate (Calbiochem), and changes in fluorescence intensity were monitored with a Cytofluor4000 (PerSeptive Biosystems, Framingham, MA) fluorescence plate reader with 360/40 excitation and 460/40 emission filters (Chroma Technology). The average reporter enzyme protein copy number per cell in CMV-β-lactamase, CMV-*lacZ*, WT, and unstimulated and stimulated (C84 NFAT-β-lactamase and C37 NFAT-*lacZ*) populations are summarized in Table 1.

Conditions for Determining Sensitivity at Low Expression Levels

Jurkat cells expressing NFAT-β-lactamase (C84 Jurkat) or NFAT-*lacZ* (C37 Jurkat) were assayed for detection of basal levels of reporter enzyme in single living cells with the use of unstimulated clonal populations. These experiments involved making measurements of detection of gene expression on cells up to 24 h after loading CCF2 or FDG substrate. A matrix of conditions was tested for both reporter systems to optimize detection of substrate conversion for each reporter or substrate system. The first set

Table 2
Dilution Protocol for Rare Event β-Lactamase Experiments[a]

Approximate frequency of positive cells	Milliliters of WT Jurkat cell suspension (10^6 cells/ml)	Volume and source of cell suspension containing CMV-β-lactamase Jurkat cells	Total no. cells in sample after all dilutions were completed
1:10	13.5	1.5 ml from CMV-β-lactamase Jurkat (10^6 cells/mL)	13.5×10^6
$1:10^2$	13.5	1.5 ml from 1:10 sample	13.5×10^6
$1:10^3$	13.5	1.5 ml from $1:10^2$ sample	13.5×10^6
$1:10^4$	13.5	1.5 ml from $1:10^3$ sample	12.5×10^6
$1:10^5$	22.5	2.5 ml from $1:10^4$ sample	15×10^6
$1:10^6$	90	10.0 ml from $1:10^5$ sample	100×10^6
0 (WT control)	100	None	100×10^6

[a]WT Jurkat cells and a clonal population of Jurkat cells constitutively expressing β-lactamase were mixed at a frequency of approximately one positive (constitutively expressing β-lactamase) per 10 cells. Tenfold dilutions were made from the 1:10 mixture into WT Jurkat cells to create mixtures of positive and WT Jurkat cells, with positive events occurring at frequencies of approximately 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$. CMV, cytomegalovirus.

of incubation conditions was with samples kept at room temperature with 2 mM of probenecid (13) in the buffer (probenecid is a blocker of nonspecific anion transport used to aid retention of fluorogenic substrate in cells over the 24-h room-temperature time course). The second set of incubation conditions was with samples kept on ice for the full time course without probenecid.

β-Lactamase. C84 Jurkat cells in log growth phase were loaded with CCF2/AM. Unstimulated C84 cells were assayed with flow cytometry (blue:green CCF2 fluorescence ratio with no fluorescence compensation) for the percentage of positive cells detected at 1, 4, 6, 18, and 24 h after loading (WT Jurkat was used as the baseline control, and stimulated C84 was used as the positive control). CCF2/AM-loaded cells were kept at room temperature with 2 mM of probenecid for detection of low-level reporter expression.

LacZ. Unstimulated C37 cells were assayed with flow cytometry (FDG-derived green fluorescence intensity) for the percentage of positive cells detected at 1, 4, 6, 18, and 24 h after hypotonic FDG loading (3) (WT Jurkat was used as the baseline control, and stimulated C37 was used as the positive control). FDG-loaded samples were kept on ice during the entire time course.

Conditions for Rare Event Detection

β-Lactamase. WT and CMV-β-lactamase Jurkat cells were loaded (separately) with CCF2/AM. Cells were mixed by using a 1:10 serial dilution to form samples of cells at approximately the following frequencies of positive cells: 1:10, $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$. Numbers of cells used for each population and the mixing protocol used are outlined in Table 2. After loading and mixing the populations, cells were centrifuged at room temperature (5 min at 207*g*), resuspended in HBS (with 0.1% bovine serum albumin and 25 mM of HEPES, pH 7.4), and kept on ice for the remainder of the experiment. Cell populations were then analyzed with flow cytometry.

LacZ. WT and CMV-*lacZ* Jurkat cells were loaded (separately) with FDG (3). After quenching the hypotonic shock of FDG loading, cells were mixed using a 1:10 serial dilution comparable to the mixing protocol outlined in Table 2 to form samples of cells at approximately the following frequencies of positive cells: 1:10, $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$ (all samples were kept on ice during dilutions). After loading and mixing the populations, cells were centrifuged at room temperature (5 min at 207*g*), resuspended in HBS (with 0.1% bovine serum albumin, 1 μg/ml of propidium iodide, and 25 mM of HEPES, pH 7.3, at 0°C), and kept on ice for the remainder of the experiment. Cell populations were then analyzed with flow cytometry. In addition to using propidium iodide (1 μg/ml) as a viability indicator, the following measures were taken to reduce the incidence of false positive events: fluorescence compensation to subtract events with high levels of cellular autofluorescence (14), the use of a "dump channel" (an irrelevant fluorescence channel used to gate out false positive events arising from burst events and electronic noise) (15–17), and gating to exclude events with higher than average yellow fluorescence and low green fluorescence to remove FDG-related false positives (2).

Statistical Comparisons of Assay Methods

To quantitate differences in the distribution of control populations for the β-lactamase and *lacZ* reporter systems, we used a signal window criterion to define the statistical separation of baseline and positive controls for both systems (18). The signal window is defined as $(m2 - m1)/[(s2 + s1)/2]$, where m2 is the mean of the positive population, m1 is the mean of the baseline population, s2 is the standard deviation of the positive population, and s1 is the standard deviation of the baseline population. The signal window increases if the separation of the mean values of the control populations increases or the standard deviations of the control populations decrease.

RESULTS

Statistical Performance of the Assay

Several statistical parameters of the *lacZ* and β-lactamase assays were analyzed and are compared in Table 3.

Table 3
*Statistical Analysis of Signal Window and Fold Change for Populations of NFAT-β-Lactamase and NFAT-lacZ Jurkat Cells**

Reporter	Fluorescence analysis method used for flow cytometry	Baseline control sample	Positive control sample	No. signal windows[a] separating positive from baseline controls	Fold change[b]
LacZ	Log scale FDG fluorescence intensity	WT Jurkat	Stimulated NFAT-lacZ Jurkat clone 37	3.4	24 ± 16
β-lactamase	Linear scale CCF2 blue and green intensities and real-time ratio (no compensation)	WT Jurkat	Stimulated NFAT β-lactamase Jurkat clone C84	31	32 ± 5
β-lactamase	Log scale CCF2 blue and green intensities (using fluorescence compensation)	WT Jurkat	Stimulated NFAT β-lactamase Jurkat clone C84	NA	482

*CCF2, Coumarin cephalosporin fluorescein; FDG, fluorescein di-β-D-galactopyranoside; NA, not available; NFAT, nuclear factor of activated T cells.

[a]The signal window is defined as $(m2 - m1)/([s2 + s1]/2)$, where m2 is the mean of the positive (stimulated) population, m1 is the mean of the baseline (WT) population, s2 is the standard deviation of the positive population, and s1 is the standard deviation of the baseline population.

[b]The fold change is the mean ratio or intensity of the positive population divided by the mean ratio or intensity of the baseline population.

We first determined the dynamic range of the assays by calculating the fold change between the positive control population (stimulated NFAT-β-lactamase [C84 Jurkat] and NFAT-*lacZ* [C37 Jurkat] clonal populations) over the baseline (WT) control population for both reporters. For NFAT-*lacZ* cells, the mean fluorescence intensity of the positive population of the stimulated control was 24 ± 16 times the background fluorescence for FDG-loaded WT Jurkat cells. For NFAT-β-lactamase cells, using linear scale fluorescence and real-time ratio for CCF2 blue and green fluorescence, the ratio for the positive population (stimulated control) was 32 ± 5 times the ratio for baseline control (WT Jurkat). The dynamic range could be extended further by compensating for spectral overlap in the blue and green channels (10% compensation green from blue and vice-versa) and using log scale fluorescence analysis. With this method the dynamic range increased to 482-fold (blue:green ratio of the positive population divided by the blue:green ratio of the baseline population). An overview of flow cytometric techniques including the use of linear and log scale fluorescence analysis for the β-lactamase/CCF2 reporter system appear as an appendix to this article.

As an aggregate measure of assay performance, we used the signal window calculation, which incorporates the dynamic range and the standard deviation of the assays, as described in Materials and Methods. When using these signal window criteria, there were 3.4 signal windows separating baseline and positive controls in the β-gal assay and 31 signal windows separating baseline and positive controls in the β-lactamase assay (linear scale fluorescence analysis and real-time ratio for CCF2 blue and green fluorescence). Signal window (statistical separation) and fold induction data for these experiments are summarized in Table 3.

Detecting Low Reporter Levels

The C84 NFAT-β-lactamase Jurkat cell line and the C37 NFAT-*lacZ* Jurkat line have very low basal levels of reporter expression in the unstimulated populations and relatively high expression levels with stimulation. To determine the ablity of the reporter systems to detect low reporter levels, we measured the appearance of a positive signal from the low-level activity of the unstimulated NFAT clones. Because of the low copy number, an extended time was required to detect conversion of the substrate. Therefore, a 24-h time course of substrate conversion was carried out on unstimulated populations of C84 and C37 Jurkat cells (Fig. 2). For CCF2/AM-loaded C84 cells, detection of low levels of reporter expression was superior after incubating samples at room temperature with 2 mM of probenecid than on ice without probenecid because of improved substrate hydrolysis kinetics at room temperature and the effective retention of the substrate inside the cells with probenecid. Inside living cells, CCF2 (after de-esterification by cellular esterases) carries a net −4 charge, and the coumarin cleavage product of CCF2 carries a net −3 charge. Active anion transport of both of these molecules out of cells is effectively blocked by probenecid. In contrast, the fluorescent hydrolysis product of FDG (fluorescein) is not well retained inside living cells with probenecid. Even though fluorescein may carry as much as a net −2 charge, the carboxylic acid group is in equilibrium with a neutral lactone conformation, allowing fluorescein to passively leave cells regardless of the presence of probenecid. For FDG-loaded C37 cells, detection of low levels of reporter expression was superior in samples on ice than in those kept at room temperature with probenecid. Also, variants of FDG including chloromethyl-FDG were tested in the current study, and none of them yielded improved results over

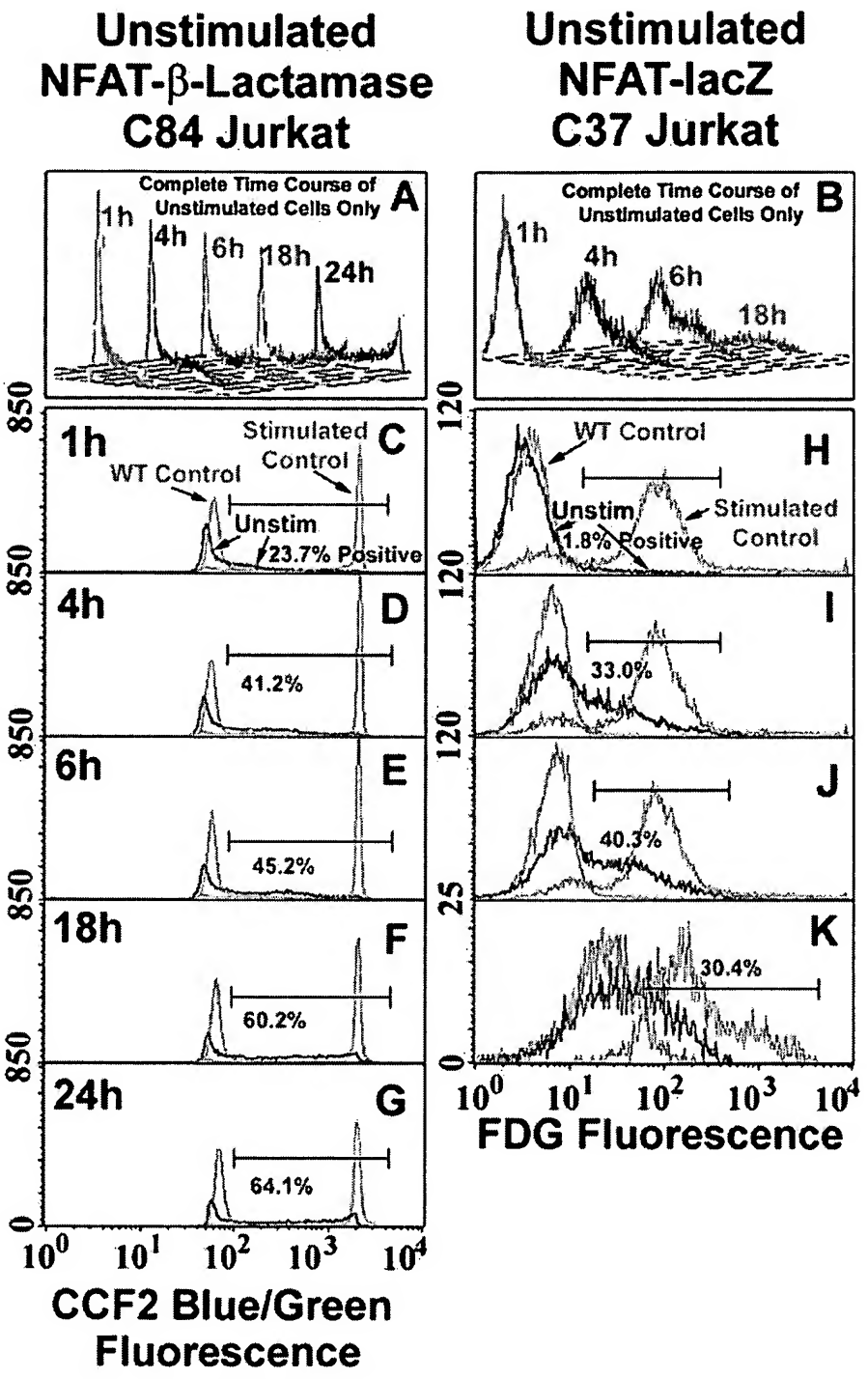

FIG. 2. Detection of low levels of reporter expression in unstimulated C84 and C37 Jurkat cells. A: Time course after loading substrate for C84 Jurkat cells. B: Time course after loading substrate for C37 Jurkat cells. C–K: Unstimulated cells (red) with WT control (green) and positive control (stimulated cells, blue) at each time point. (Percent positive detected in unstimulated populations indicated for each time point.)

FDG. At the 1-h time point, about 24% of the unstimulated NFAT-β-lactamase cells were detected above the Gaussian distribution of the WT population, whereas only about 2% of the unstimulated NFAT-*lacZ* cells were detected above the same control in the β-gal assay. The percentage of positive cells detected in the unstimulated population in the β-gal assay reached a peak at the 6-h time point (around 40% of cells detected) and subsequently declined, probably as a result of substrate leakage. In contrast, the β-lactamase readout continued to register cells (up to about 80% positive) throughout the 24-h time point due to the improved retention of the substrate (cells kept at room temperature). This conversion was due to transfected reporter because WT cells showed no conversion over the same period.

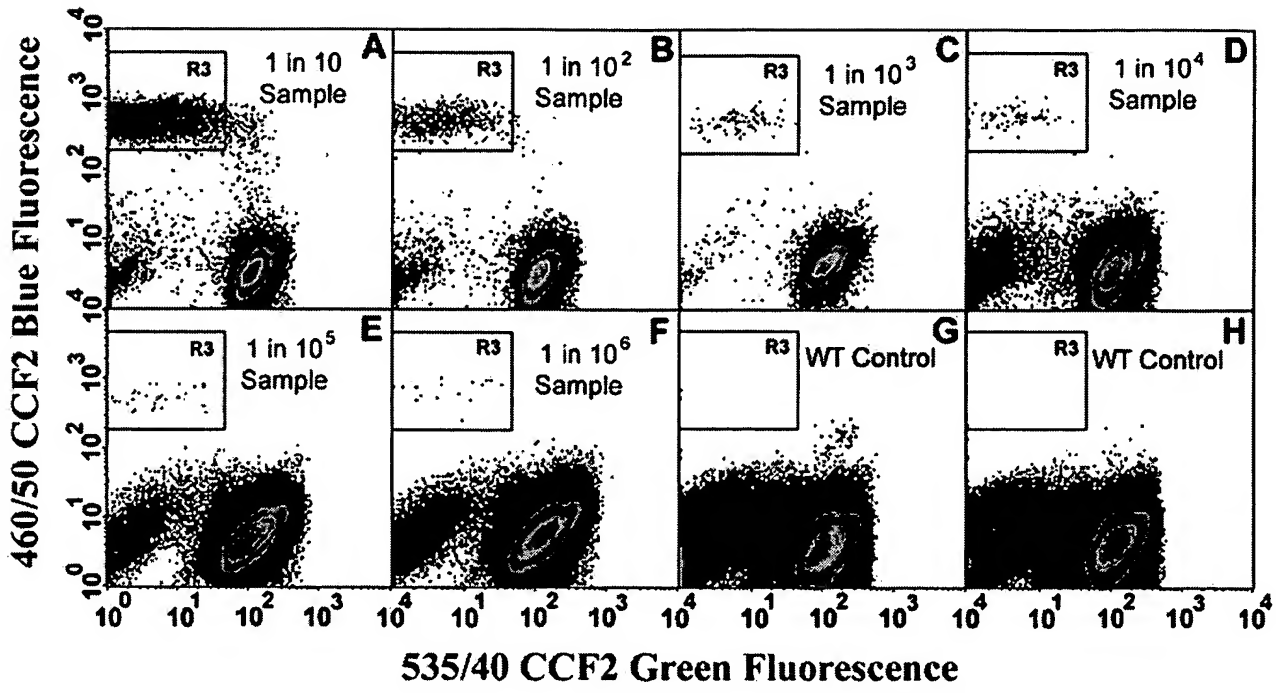

FIG. 3. Detection of rare events using β-lactamase/coumarin cephalosporin fluorescein (CCF2). R3 defines positive events. A: Positive event frequency of approximately 1:10 from a 100,000 event data file. B: $1:10^2$ from 100,000 events. C: $1:10^3$ from 100,000 events. D: $1:10^4$ from 1,000,000 events. E: $1:10^5$ from 5,000,000 events. F: $1:10^6$ from 20,000,000 events. G: WT control from 20,000,000 events. H: Duplicate of WT control.

Detection of Rare Events

For rare event experiments, CCF2 was analyzed using log scale fluorescence intensities with fluorescence compensation (10% green from blue and vice-versa). (An overview of flow cytometric techniques including the use of linear and log scale fluorescence analysis for the β-lactamase/CCF2 reporter system appear as the appendix.) To accurately determine the percentage of positive cells that had been mixed into a population of WT cells, the sample with the highest frequency of positive events (mixed at approximately one positive cell in 10) was used as the standard for subsequent dilutions (i.e., this frequency was used to define the expected number of positive cells for subsequent dilutions). The total number of events for each data file ranged from 100,000 (for the 1:10 sample) to 20×10^6 (for the $1:10^6$ samples and WT control samples). A total of 100×10^6 events were analyzed for WT only and for the $1:10^6$ samples over the course of two experiments. CMV-β-lactamase Jurkat cells (positive events for β-lactamase rare event detection) were detected at nearly the expected frequency for all dilutions up to a frequency of $1:10^6$ (highest dilution tested). Density plots of CCF2 blue versus green fluorescence (log scale using fluorescence compensation) for β-lactamase rare event experiments are displayed in Figure 3. Positive events are clearly visible in the R3 region for all dilutions. In the 20 million event data files for the WT control (20×10^6 events/data file), an average of two false-positive gated events were detected per file, for a false positive frequency of about $1:10^7$.

Rare events expressing β-gal were detected reliably up to positive event frequencies of about $1:10^4$. At a positive event frequency of $1:10^5$, false positive events outnumbered true positive events by twofold. At a frequency of $1:10^5$, the appearance of a positive "peak" became obscured by false positives (compare with the WT control in Figure 4). In a representative β-gal rare event experiment with about 5×10^6 cells per sample, there were 43 positive events expected in the $1:10^5$ sample (based on dilution of the 1:10 control sample), and an average of 108 was detected. In the same experiment, an average of 67 positive events was detected in the WT control (no cells expressing β-gal). Subtracting the false positive event rate from the detected number of positive events in the $1:10^5$ sample yielded very close to the expected number of true positive events. These results suggested that the true positive events are in fact being detected, but that, at this frequency of positive events, the true positive events become mixed with a higher fraction of false positives. The percentages of expected positive events detected are plotted against the positive event frequency for both reporter systems in Figure 5. Detection of more than 100% of the expected positive events arose from false positives.

DISCUSSION

These results demonstrate that the β-lactamase reporter system is highly sensitive and has a very low incidence of false positive events in flow cytometric assays. CCF2 has advantages over FDG as a reporter substrate in terms of

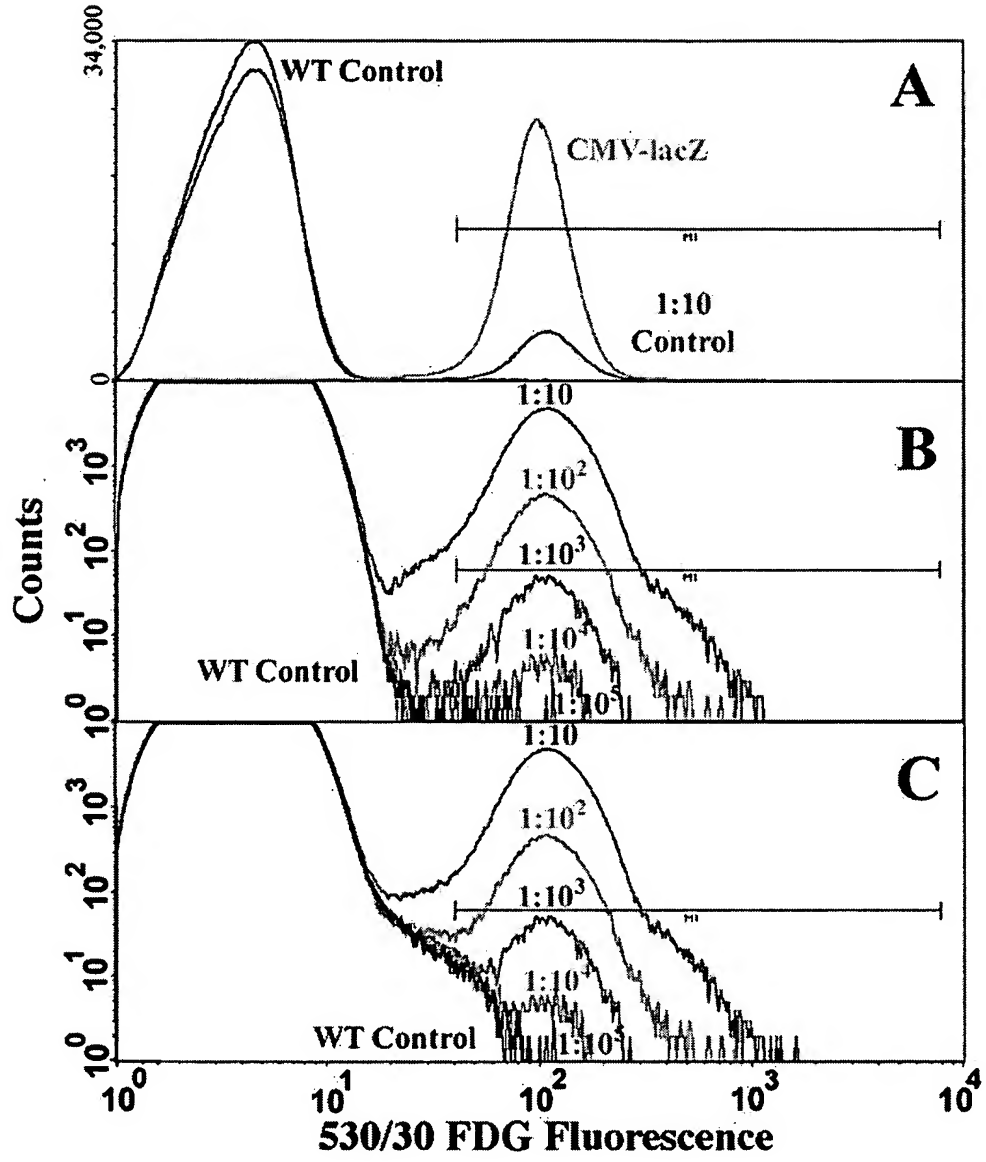

FIG. 4. Detection of rare events using *lacZ*/fluorescein di-β-D-galactopyranoside (FDG). A: Positive event frequency of 1:10, with WT and cytomegalovirus (CMV)/*lacZ* controls (linear vertical axis for visualization of all). B: Positive event frequencies of approximately 1:10, $1:10^2$, $1:10^3$, $1:10^4$, and $1:10^5$ (log scale vertical axis for wide range of frequencies). Gating includes yellow fluorescence to eliminate false positives arising from FDG. C: Same as B except that gating on yellow fluorescence is not used.

experimental convenience and assay performance. From a convenience perspective, CCF2 simplifies experimental procedures because it is cell permeant (CCF2/AM) and well retained by cells (some cell types, such as CHO-K1 cells, actively pump out anionic dyes such as CCF2, and in these cases, incubation of cells with probenecid, an anion transport inhibitor, is recommended). Thus, a hypotonic shock step for loading and keeping cells on ice before the assay are not required. Although FDG can be incubated at room temperature, the free fluorescein generated by the β-gal reaction rapidly leaks from cells and is not well retained by probenecid. The issue of substrate retention is aggravated further by the active extrusion of fluorescein-galactoside by certain cell types (8).

In terms of performance, the ratiometric readout of β-lactamase improves statistical separation of baseline and positive populations and therefore improves the resolution of positive rare events and reduces false positives. One reason for this improvement is that, by calculating a ratio of two wavelengths, variations in cell size and substrate concentration from cell to cell are effectively normalized. Under most conditions both wavelengths are well above background, thus minimizing the contribution of cellular autofluorescence at low gene expression levels.

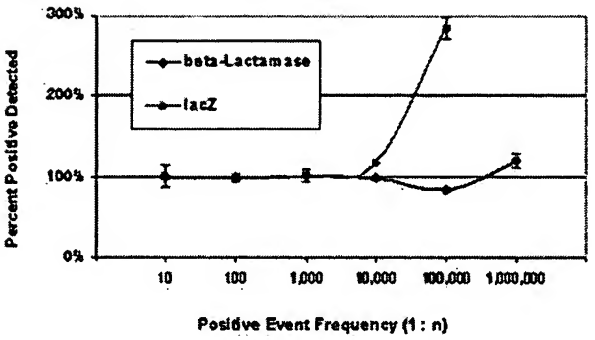

FIG. 5. Percentage of positive events detected versus expected positive event frequency. Rare event detection for mixed populations of WT and cytomegalovirus (CMV) β-lactamase Jurkat cells and mixed populations of WT and CMV-*lacZ* Jurkat. Detection of greater than 100% of the expected number of positive cells arises from false positives.

This is an important factor when comparing the β-lactamase and the β-gal systems because cellular autofluorescence contributes significantly to the signal in the low expressing cells, as shown by the higher population coefficients of variation (14% vs. 42% for β-lactamase and β-gal systems, respectively).

The performance difference between the β-lactamase and β-gal systems could be quantitatively observed when using matched cell lines expressing the same reporter constructs. By using a signal window calculation for the NFAT inducible reporter as an aggregate statistical measure of assay quality, we found that the signal window was approximately 10-fold higher (3.4 vs. 31) for the β-lactamase assay. This would be expected to improve the detection of rare events. We confirmed this by performing experiments in which we spiked WT populations with reporter-expressing cells. It has been previously reported that false positive events are prone to occur at a frequency in the range of 0.01–1% in the β-gal assay (2). By using the techniques documented in this work to avoid the interference of false positives in the β-gal readout, we observed false positive frequencies in the range of $1:10^4$ to $1:10^5$, in general agreement with previous findings. By using similar methods for the β-lactamase-expressing cells, we consistently observed false positive rates in the β-lactamase experiments at a frequency of about $1:10^7$, an improvement of greater than two orders of magnitude in the instance of false positive events. Interestingly, at these frequencies, the main limitation in rare event sorting becomes the throughput of the sorter, not the reliability of the indicator.

One consideration in using the β-lactamase system is that the coumarin moiety of the CCF2 substrate is optimally excited when using violet wavelengths (400–415 nm) and that the FRET assay presents the opportunity for applying compensation (a the form of background subtraction) to increase the fold change of positive over negative populations. The advent of inexpensive diode lasers no doubt will facilitate the use of the β-lactamase system for high-end sorting and benchtop scanning. We reported the use of a violet diode laser on a BD LSR system with little performance loss compared with the more expensive krypton or mixed-gas lasers.

Although the *lacZ*/FDG system has been a quite useful and powerful assay for single-cell gene expression measurements, the advantages of the β-lactamase system will facilitate new applications difficult to achieve with previous technologies. For example, the rare event capabilities can be used to help scan genomic libraries for genes responsive to a particular stimulus. The sensitivity at low expression levels can be useful for identifying or studying genes with low levels of expression or downregulated genes. These capabilities were demonstrated by Whitney et al. (10) who identified low-expressing and downregulated genes from genomic cell libraries containing several million clones. Future applications taking advantage of these capabilities could include directed evolution of genes or cell signaling elements.

APPENDIX: FLOW CYTOMETRIC TECHNIQUES AND EXCITATION LASERS FOR β-LACTAMASE/CCF2 SORTING AND ANALYSIS

One can use two techniques for analyzing and sorting cells based on the β-lactamase/CCF2 reporter system. One technique uses log scale fluorescence analysis and fluorescence compensation. On some flow cytometers, this technique has the advantage that pulse processing is not required, and the instrument's dead time is minimized, thereby enabling higher sort throughput. The other technique uses linear scale fluorescence analysis, no fluorescence compensation, and real-time ratio analysis in a manner analogous to that typically used for Indo-1 (19). Violet (typically 407 nm or 413 nm from a krypton laser) or UV excitation can be used for CCF2.

For comparison of excitation wavelength for CCF2, a mixture of WT and CMV-β-lactamase Jurkat cells loaded with CCF2 were analyzed on a BD FACSVantage SE using 45 mW of violet (413 nm) excitation (Fig. 6A–D) or 45 mW of UV (351–357 nm) excitation (Fig. 6E–H). The same mixture of cells was also analyzed on a customized BD LSR benchtop flow cytometer modified with a 5-mW violet (404 nm) diode laser (Fig. 6I–L). Differences in separation of blue:green ratio of baseline (WT Jurkat) and positive (CMV-β-lactamase) populations for each excitation source are summarized in Table 4. It is clear that violet excitation (with optimized collection optics on the FACSVantage SE) yields the greatest statistical separation of the control populations. There are two principal reasons that the violet excitation of CCF2 is more efficient than UV excitation. One is the close proximity of the violet wavelength (413 nm) to the excitation maximum (409 nm) of the donor molecule (coumarin) in CCF2. In addition, a great deal of cellular autofluorescence in the blue channel is reduced by avoiding UV excitation (20), and the improved separation of the blue:green ratio of control populations is due mostly to the improved signal over background in the blue channel. The separation of the blue:green ratio of control populations with the violet diode laser as the excitation source is less than would be anticipated for a

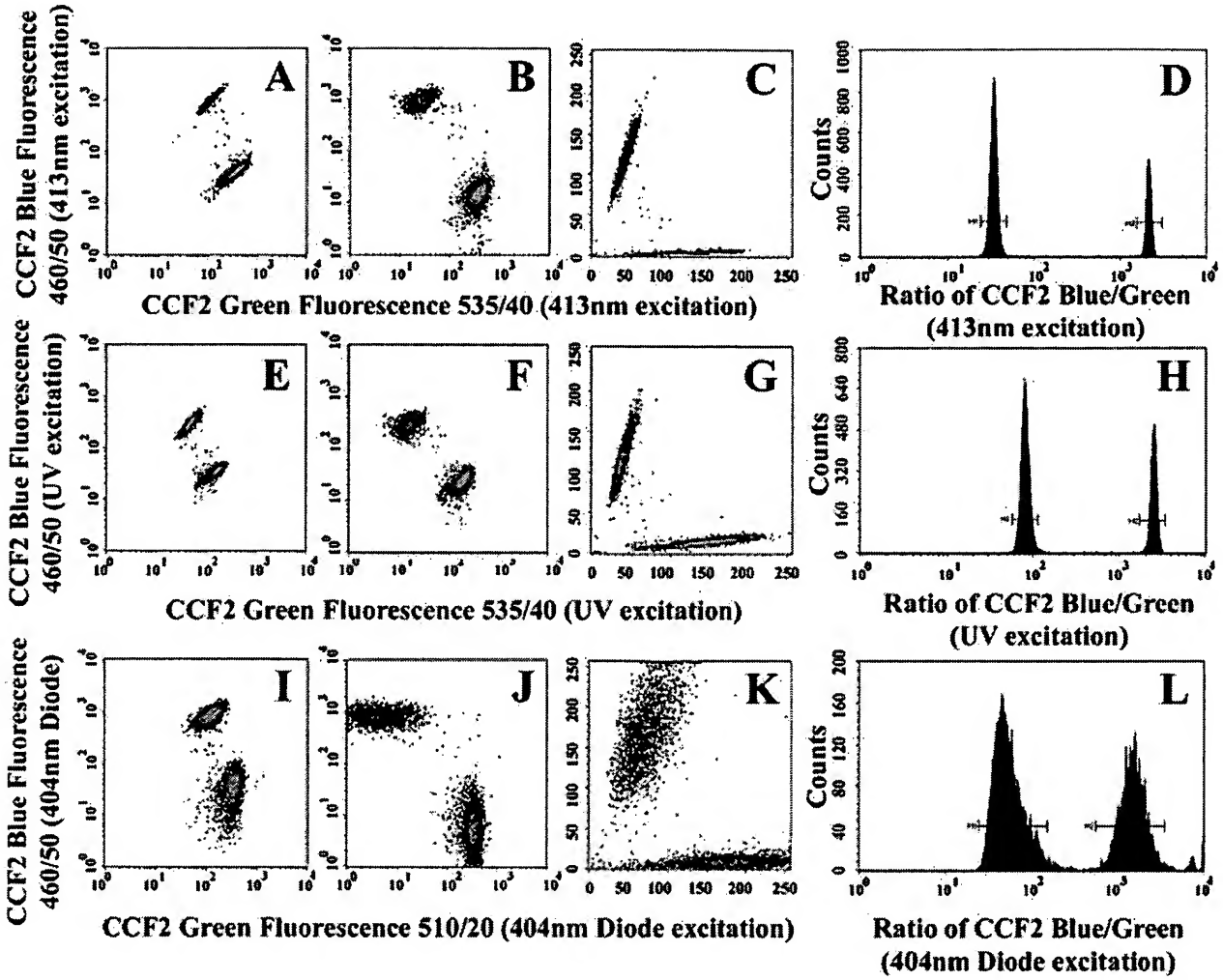

FIG. 6. Comparison of excitation wavelengths and analysis techniques for coumarin cephalosporin fluorescein (CCF2). WT and cytomegalovirus (CMV) β-lactamase Jurkat cells loaded with CCF2/AM were analyzed as follows. A–D: Becton Dickinson (BD) FACSVantage, 45 mW of 413-nm excitation. A: Log scale, no compensation. B: Log scale, 10% compensation. C: Linear scale intensities. D: Real-time ratio of blue:green from intensities in C. E–H: Same as A–D except that ultraviolet (351–364 nm) excitation instead of 413-nm excitation was used. I–L: Modified BD LSR, 5 mW of 404-nm violet excitation from diode laser. I: Log scale, no compensation. J: Log scale, 18% compensation. K: Linear scale intensities. L: Real-time ratio of blue:green from intensities in K.

Table 4
*Statistical Analysis of Fold Change and Signal Window for a Mixed Population of WT and CMV-β-Lactamase Jurkat Cells Comparing Different Excitation Wavelengths and Analysis Techniques for CCF2**

		Blue:green ratio			
Wavelength/instrument	Analysis mode	WT	CMV-β-lactamase	Fold change (CMV-β-lactamase/WT)	Signal window
Violet (413 nm)/ FACSVantage SE	Real-time ratio	34	2140	63	31.5
	Log 0% compensation	0.13	9.2	71	NA
	Log 8% compensation	0.05	38	760	NA
Ultraviolet (351–357 nm)/FACSVantage SE	Real-time ratio	83	2510	30	22.0
	Log 0% compensation	0.24	6.5	27	NA
	Log 10% compensation	0.15	19	127	NA
Diode (404 nm)/LSR	Real-time ratio	58	1590	27	5.5
	Log 0% compensation	0.13	7.4	57	NA
	Log 18% compensation	0.03	150	5,000	NA

*CCF2, coumarin cephalosporin fluorescein; CMV, cytomegalovirus; NA, not available.

violet excitation source due to the suboptimal emission filter configuration available for CCF2 analysis on the BD LSR, poor beam shape, and power available from this 5-mW diode laser. However, these experiments demonstrated a novel application for violet diode lasers in flow cytometry, and with ongoing improvements in diode laser technologies and capabilities, their use may be more prevalent in the future.

Using log scale fluorescence intensities and fluorescence compensation allows for two-dimensional visualization of the fluorescence distribution of a population and has potential throughput advantages. In practice, 10% fluorescence compensation blue from green and vice-versa is reasonable to apply because of the spectral overlap of the cleaved and uncleaved forms of CCF2. As fluorescence compensation is increased, the blue:green ratio of WT Jurkat cells decreases, and the blue:green ratio of CMV-β-lactamase Jurkat cells increases. With fluorescence compensation turned on (8% for 413-nm violet, 10% for UV, and 18% for the LSR configuration), separation of the blue:green ratio of CMV-β-lactamase Jurkat cells over the blue:green ratio of WT Jurkat cells is dramatically increased (Fig. 6B,F,J and Table 4).

When using real-time ratio and linear scale fluorescence intensities for the blue and green fluorescence, the blue:green ratio of CMV-β-lactamase Jurkat cells over the blue:green ratio of WT Jurkat cells is greatest with the 413-nm violet excitation source. When using the UV excitation source or the lower power 5-mW 404-nm violet excitation source, the ratio change drops by a factor of approximately 2 (Fig. 6C,D,G,H,K,L and Table 4).

Overall, violet excitation of CCF2 provides superior performance of the fluorescence readout, although UV excitation can also be used very effectively. We demonstrated that the β-lactamase/CCF2 reporter readout may be used with log scale fluorescence analysis with fluorescence compensation or with linear scale fluorescence analysis with real-time ratio. We also demonstrated the proof of principle for application of the β-lactamase/CCF2 reporter readout with the BD LSR benchtop cytometer and with a violet diode laser excitation source.

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